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Environmental Surveillance of Norovirus Genogroups I and II for Sensitive Detection of Epidemic Variants

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ABSTRACT Sewage samples have been investigated to study the norovirus concentrations in sewage or the genotypes of noroviruses circulating in human populations. However, the statistical relationship between the concentration of the virus and the number of infected individuals and the clinical importance of genotypes or strains detected in sewage are unclear. In this study, we carried out both environmental and clinical surveillance of noroviruses for 3 years, 2013 to 2016. We performed cross-correlation analysis of the concentrations of norovirus GI or GII in sewage samples collected weekly and the reported number of gastroenteritis cases. Norovirus genotypes in sewage were also analyzed by pyrosequencing and compared with those identified in stool samples. The cross-correlation analysis found the peak coefficient ($R = 0.51$) at a lag of zero, indicating that the variation in the GII concentration, expressed as the $\log_{10}$ number of copies per milliliter, was coincident with that in the gastroenteritis cases. A total of 15 norovirus genotypes and up to 8 genotypes per sample were detected in sewage, which included all of the 13 genotypes identified in the stool samples except 2. GII.4 was most frequently detected in both sample types, followed by GII.17. Phylogenetic analysis revealed that a strain belonging to the GII.17 Kawasaki 2014 lineage had been introduced into the study area in the 2012-2013 season. An increase in GI.3 cases was observed in the 2015-2016 season, and sewage monitoring identified the presence of GI.3 in the previous season (2014-2015). Our results demonstrated that monitoring of noroviruses in sewage is useful for sensitive detection of epidemic variants in human populations.

IMPORTANCE We obtained statistical evidence of the relationship between the variation in the norovirus GII concentration in sewage and that of gastroenteritis cases during the 3-year study period. Sewage sample analysis by a pyrosequencing approach enabled us to understand the temporal variation in the norovirus genotypes circulating in human populations. We found that a strain closely related to the GII.17 Kawasaki 2014 lineage had been introduced into the study area at least 1 year before its appearance and identification in clinical cases. A similar pattern was observed for GI.3; cases were reported in the 2015-2016 season, and closely related strains were found in sewage in the previous season. Our observation indicates that monitoring of noroviruses in sewage is useful for the rapid detection of an epidemic and is also sensitive enough to study the molecular epidemiology of noroviruses. Applying this approach to other enteric pathogens in sewage will enhance our understanding of their ecology.

KEYWORDS Norovirus, massive parallel sequencing, molecular epidemiology, rapid detection of epidemic, sewage, virological surveillance

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Noroviruses are among the leading etiological agents of viral gastroenteritis across all ages. Infection can be fatal in vulnerable populations such as infants and the elderly, although asymptomatic infections are also common (1). Among patients hospitalized for gastroenteritis and those visiting a clinic, norovirus is the most common cause of diarrhea in adults and the second most common cause (after rotavirus) in children (1). Noroviruses globally cause approximately 700 million illnesses and 0.2 million deaths per year, leading to $60 billion in social losses, including direct health care costs and productivity losses (2).

Noroviruses are positive-sense single-stranded RNA viruses belonging to the family *Caliciviridae*. The genome includes three open reading frames (ORFs). ORF1 encodes the nonstructural polyprotein, including the RNA-dependent RNA polymerase (RdRp). ORF2 and ORF3 encode the two structural proteins, the major (VP1) and minor (VP2) capsid proteins, respectively (3). VP1 consists of an N-terminal domain, a shell (S) domain, and a protruding (P) domain, which is further divided into the P1 and P2 subdomains (4). Noroviruses are classified into seven genogroups, and each genogroup is further divided into genotypes based on the phylogenetic clustering of the complete VP1 amino acid sequences (5). Genogroup I (GI), GII, and GIV are human noroviruses and are further classified into 9, 22, and 2 genotypes (1 GIV genotype for humans), respectively (5). GII genotypes have been detected in clinical cases more frequently than GI, whereas GIV has been rarely detected (6, 7). Since the mid-1990s, outbreaks and sporadic cases of genogroup II genotype 4 (GII.4) have frequently been reported (1, 8) and its different variants have emerged approximately every 2 to 3 years (5). Recently, a new GII.17 variant known as Kawasaki 2014 emerged in Asia. This variant has caused an outbreak and sporadic cases across the world, replacing previously prevalent GII.4 Sydney 2012 (9–14). Since novel variant strains may be latent in a community as minorities before producing an epidemic (15), it is necessary to understand the diverse genotypes of noroviruses circulating in human populations.

The number of gastroenteritis cases caused by noroviruses has a seasonal peak in the winter months in temperate regions of Europe and North America (16), as well as in Japan (17). Since sewage theoretically receives noroviruses excreted in feces or vomitus from all of the infected individuals in a catchment area, it has been investigated as one of the comprehensive approaches to study the molecular epidemiology of this virus (18–20). However, limited studies have investigated both environmental and stool samples, and the cloning-sequencing approach employed has identified only the predominant genotypes or strains in sewage (21–23). Very recently, a pyrosequencing approach has been employed to investigate noroviruses in sewage, and multiple genotypes circulating in human populations were identified (24, 25). Our previous study succeeded in providing detailed information regarding the temporal dynamics of noroviruses (25); however, that study was conducted in a single epidemic period and a long-term study was necessary to evaluate the sensitivity and applicability of this approach.

Recently, some studies have attempted to utilize the concentration of human enteric viruses in sewage to estimate the prevalence of infections in human populations. Lodder et al. demonstrated that the concentration of poliovirus (oral attenuated live polio vaccine) in sewage well reflected the number of individuals shedding the virus (26). Other studies showed that the highest concentration of norovirus in sewage was observed at a time similar to that of a peak in norovirus gastroenteritis cases (23, 27, 28). However, those studies were carried out for a limited period of time and the statistical relationship between the two time series data, the norovirus concentration in sewage and the number of gastroenteritis cases, is unknown.

In the present study, we performed both environmental and clinical surveillance of noroviruses over 3 years. Norovirus GI and GII concentrations in sewage samples collected weekly were determined by real-time quantitative PCR (qPCR) assays, and these genotypes were investigated by pyrosequencing. To evaluate the relationship between the norovirus concentration in sewage and the number of gastroenteritis cases in the area, we performed cross-correlation analysis by using the concentration of

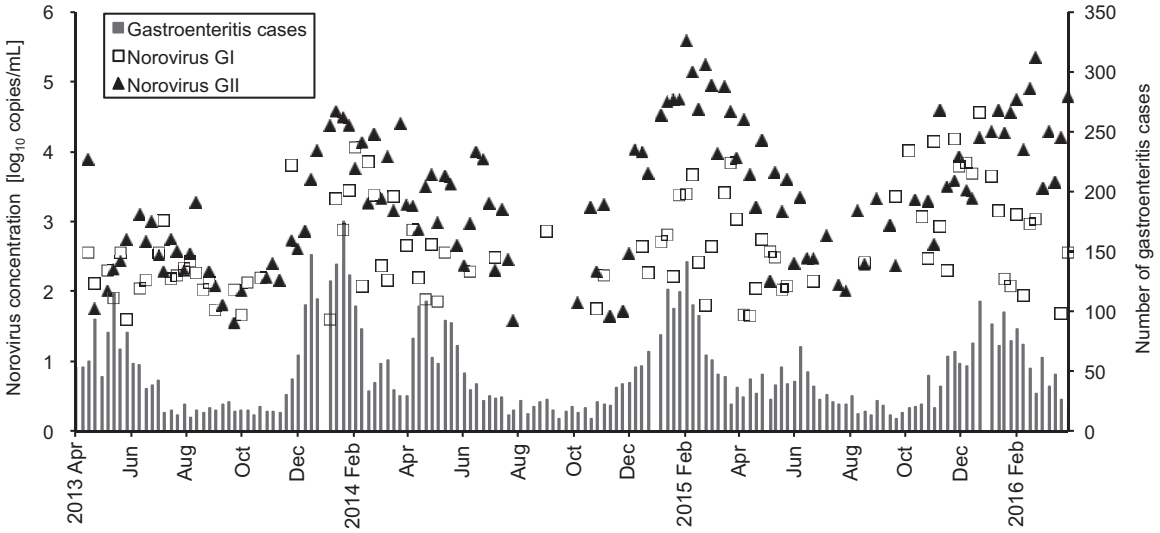

FIG 1 Concentrations of norovirus GI and GII in sewage and numbers of gastroenteritis cases reported in the study area. The norovirus GI and GII detection limits are 1.5 and 1.6 $\log_{10}$ copies/ml, respectively.

norovirus GI or GII in sewage and the number of gastroenteritis cases reported in the study area. In parallel, we also determined the norovirus genotypes detected in gastroenteritis cases and compared the temporal prevalence and diversity of norovirus genotypes in both sewage and stool samples.

RESULTS

Norovirus concentrations in sewage and reported gastroenteritis cases. The overall rates of recovery of the murine norovirus (MNV) used to spike sewage samples ranged from 1.4 to 45% (geometric mean, 13%; $n = 154$), suggesting that there was no significant loss of viruses in the polyethylene glycol (PEG) precipitation or inhibition in reverse transcription (RT)-qPCR. This result allowed us to quantify the norovirus concentrations in the sewage samples. Figure 1 shows the concentrations of norovirus GI and GII in sewage and the number of gastroenteritis cases reported in the study area based on the pediatric sentinel surveillance. Both the concentrations of noroviruses and the number of gastroenteritis cases increased in the winter months, from November to February, and decreased in the summer months, from June to August. These trends were observed similarly in each norovirus season (i.e., from September to August).

In the 2013-2014 and 2014-2015 norovirus seasons, GI was sporadically detected in sewage, with relatively low concentrations from September to December. After that, the concentrations increased to 1.1×10^4 copies/ml in February 2014 and 6.9×10^3 copies/ml in March 2015, respectively, and then decreased until June. In July and August, GI was rarely detected. In contrast, in the 2015-2016 season, GI was continuously detected in sewage from October and the concentrations in November and December (up to 3.5×10^4 copies/ml) were higher than those in the following 3 months.

GII was continuously detected in sewage in the 2013-2014 season, with two peaks from September to July. The concentration increased to 4.0×10^4 copies/ml in January and then decreased but again increased from April to June. In the 2014-2015 season, GII was continuously detected in sewage from October to August, with a maximum concentration of 3.9×10^5 copies/ml in February. In contrast to the previous season, GII was frequently detected between July and August. In the 2015-2016 season, GII was detected from September on and the maximum concentration (2.2×10^5 copies/ml) was observed in February, as in the previous season.

Cross-correlation analysis of the norovirus concentrations in sewage and the reported gastroenteritis cases. When the cross-correlation analysis found the peak coefficient with a lag of zero, plus, or minus, this indicated that the variation in the virus

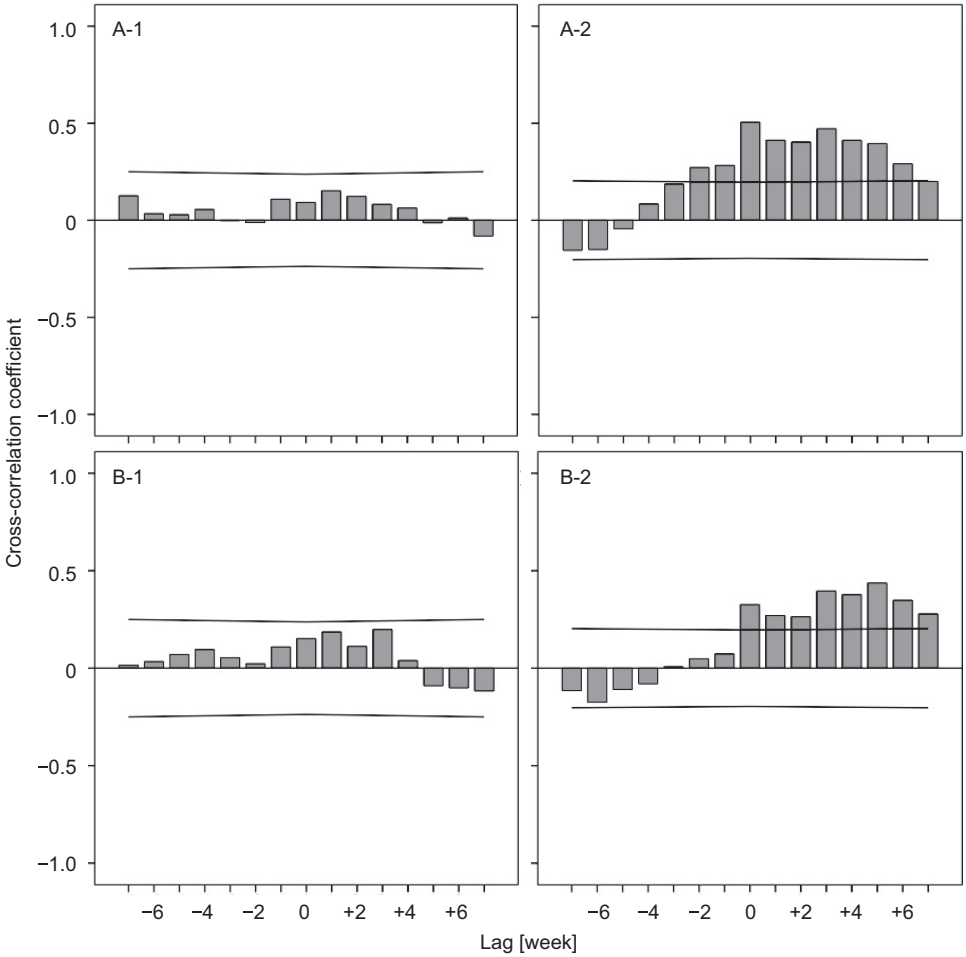

FIG 2 Distribution of cross-correlation coefficients between the number of gastroenteritis cases reported in the study area (A) or in Japan (B) and the concentration of norovirus GI (A-1 and B-1) or GII (A-2 and B-2) in sewage. The lag of the time that the norovirus concentration changed behind the time that the number of gastroenteritis cases changed was defined as "+." The two horizontal lines represent the 95% confidence interval for the correlation.

concentration, expressed as the $\log_{10}$ number of copies per milliliter, was coincident with, behind, or ahead of the variation in the number of gastroenteritis cases. No significant correlation between the norovirus GI concentration in sewage and the number of gastroenteritis cases reported in the study area or in Japan was observed (Fig. 2A-1 and B-1). In contrast, the GII concentration, expressed as the $\log_{10}$ number of copies per milliliter, significantly correlated with the number of gastroenteritis cases reported in the study area with a lag of -2 to $+6$ weeks or in Japan with a lag of 0 to $+7$ weeks (Fig. 2A-2 and B-2). The highest correlation coefficient was observed with a lag of 0 weeks for the study area ($R = 0.51$) or with a lag of $+5$ weeks for Japan ($R = 0.44$), suggesting that the concentration of norovirus GII in sewage changed synchronously with the number of infected individuals in the catchment area or changed with a 5-week delay with respect to the number of infected individuals in Japan.

Virological surveillance for gastroenteritis cases. In total, 296 rectal swab samples were collected from patients with gastroenteritis from the 14th week of 2013 to the 13th week of 2016. Of these samples, 102 (34%) were positive for norovirus(es), including 14 cases of GI infection (13%), 92 cases of GII infection (87%), and 4 cases of GI and GII coinfection (4%). Other viruses were detected in 52 of the norovirus-negative samples and 4 of the norovirus-positive samples, namely, rotavirus (33 cases), sapovirus (13 cases), astrovirus (8 cases), adenovirus (1 case), and parechovirus (1 case).

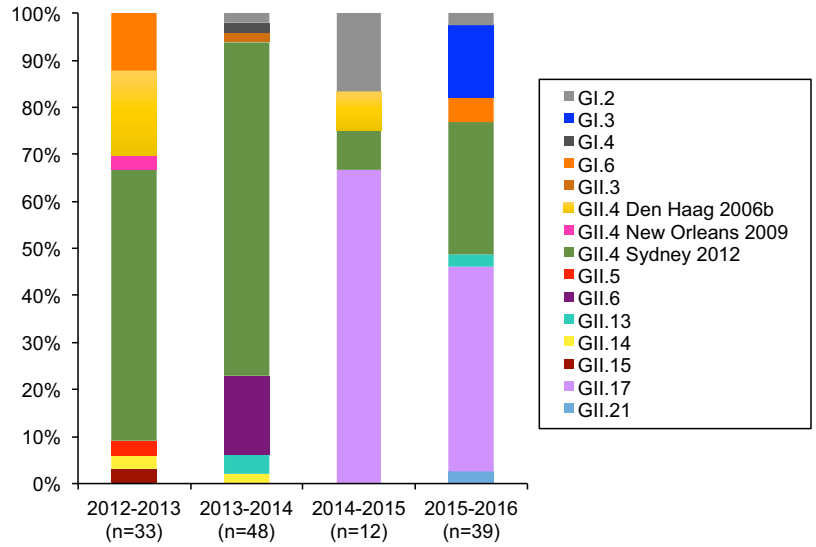

FIG 3 Genotypes identified in clinical samples in each norovirus season. The period of each norovirus season is September to August, except in the 2012-2013 (November to August) and 2015-2016 (September to March) seasons.

In this study, 101 nucleotide sequences were obtained from norovirus-positive stool samples and 12 genotypes were identified, including four GI genotypes (GI.2, GI.3, GI.4, and GI.6) and eight GII genotypes (GII.3, GII.4, GII.6, GII.13, GII.14, GII.15, GII.17, and GII.21). Norovirus GII.4 was the genotype most frequently detected during the present study period (48 cases, 48%), followed by GII.17 (25 cases, 25%), GII.6 (8 cases, 8%), GI.3 (6 cases, 6%), GI.2 (4 cases, 4%), and other genotypes. GII.4 included two variants, Sydney 2012 (47 cases) and Den Haag 2006b (1 case).

Figure 3 shows the genotypes identified in stool samples in each norovirus season, which includes genotypes identified in stool samples collected during the previous study period (November to March of the 2012-2013 season) to confirm the variation of genotypes in each norovirus season.

Sewage sample analysis by pyrosequencing. Twenty-three sewage samples were analyzed by pyrosequencing, and norovirus GI and GII were detected in 20 and 22 samples, respectively. After the denoising of nucleotide sequences and chimera removal (see the bioinformatic analysis section), 827,433 sequence reads were obtained as norovirus reads, with a mean of 22,374 reads for GI and 17,270 for GII per sample. The mean number of OTUs per sample was 5.0 for GI and 13 for GII. On the basis of the number of OTUs, GII exhibited a higher diversity than GI in wastewater samples ($P = 0.014$, Wilcoxon signed-rank test). Most rarefaction curves reached or almost reached a plateau (17/20 samples for GI and 17/22 samples for GII), which indicates that those pyrosequencing runs were deep enough to confirm the diversity of noroviruses. The other runs (i.e., June 2014 and April and August 2015 for GI and October and December 2013, June and October 2014, and August 2015 for GII) did not reach a plateau, partly because norovirus concentrations, as well as the diversity of noroviruses in sewage samples, were quite low (geometric mean, 60 copies/ml).

Comparison of norovirus genotypes detected in stool and sewage samples. Table 1 shows the norovirus genotypes detected in the stool and sewage samples in each norovirus season during the present and previous study periods. We found 15 norovirus genotypes in sewage samples (6 for GI and 9 for GII) in the 2012-2013 season, 13 (5 for GI and 8 for GII) in the 2013-2014 season, 10 (3 for GI and 7 for GII) in the 2014-2015 season, and 11 (4 for GI and 7 for GII) in the 2015-2016 season. All of the genotypes detected in stool samples were also detected in sewage samples in the corresponding season, except in two cases (GII.15 in the 2012-2013 season and GII.21 in the 2015-2016 season).

TABLE 1 Distribution of norovirus genotypes detected in stool and sewage samples in each norovirus season[a]

	GI genotype							GII genotype																
										GII4														
Season	GI1	GI2	GI3	GI4	GI6	GI7	GI9	GII2	GII3	Den Haag 2006b		New Orleans 2009		Sydney 2012	GII5	GII6	GII7	GII8	GII12	GII13	GII14	GII15	GII17	GII21
2012–2013	○	○	○	○	●	○		○		●		●		●	○	●	○		○	○	●	◎	○	
2013–2014		●	○	●	○		○		●					●		●	○	○		●	●		○	
2014–2015		●	○				○	○	○	●				●		○				○	○		●	
2015–2016	○	●	●		●			○	○					●		○				●	○		●	◎

[a]Symbols: ●, genotypes detected in both sewage and stool samples; ○, genotypes detected in sewage samples; ◎, genotypes detected only in stool samples.

Four GI genotypes (GI.2, GI.3, GI.4, and GI.6) were detected in both stool and sewage samples. GI.2 was detected in the stool samples of all of the norovirus seasons except 2012-2013, whereas it was found in the sewage samples of all four seasons. GI.3 was detected only in stool samples in the 2015-2016 season but was found in sewage in all of the seasons. GI.4 was detected in the stool samples of the 2013-2014 season and in the sewage samples of the 2012-2013 and 2013-2014 seasons. GI.6 was detected only in stool samples in the 2015-2016 season and in sewage samples in all of the norovirus seasons except 2014-2015. GI.1 and GI.9 were detected only in sewage samples in two seasons each, seasons 2012-2013 and 2015-2016 and seasons 2013-2014 and 2014-2015, respectively.

GII.4 was the predominant genotype in the stool samples in the first two seasons (i.e., 2012-2013 and 2013-2014) (Fig. 3) and was also found in sewage (Table 1). The same GII.4 variants emerged in both stool and sewage samples in each season; the Den Haag 2006b variant was detected in the 2012-2013 and 2014-2015 seasons, the New Orleans 2009 variant was found only in the 2012-2013 season, and the Sydney 2012 variant was found in all of the seasons. GII.17 first appeared in a sewage sample in the 2012-2013 season (the sample collected in January 2013) and was detected in all of the seasons. This genotype was predominant in clinical cases in the last two seasons (i.e., 2014-2015 and 2015-2016) (Fig. 3). GII.6 was detected in clinical cases in the 2012-2013 and 2013-2014 seasons, but it was found in sewage in every norovirus season. GII.3, GII.13, and GII.14 were observed less often in clinical cases; however, they were detected in sewage samples in all four norovirus seasons except the 2012-2013 season for GII.3. The other GII genotypes (GII.2, GII.5, GII.7, GII.8, and GII.12) were detected only in sewage samples.

Phylogenetic analysis of norovirus strains detected in stool and sewage samples. Phylogenetic trees of the RdRp-N/S region of strains obtained in the present and previous studies (25) were constructed for each genotype by the neighbor-joining method. Each tree showed a strong clustering of the norovirus strains detected in stool and sewage samples with near collection dates, indicating that noroviruses detected in sewage reflect the strains circulating in the catchment area. Among the genotypes detected in both sewage and stool samples, GII.17 and GI.3 strains detected in the present and previous studies formed two distinct clusters in each tree (Fig. 4). The GII.17 strains formed subcluster Kawasaki323 2014 and subcluster Kawasaki308 2015 (10) (Fig. 4A). The GII.17 strains detected in the sewage samples of January 2014 (140108_S), as well as January and February 2013 (130116_S, 130123_S, 130130_S, and 130206_S), were included in subcluster Kawasaki323 2014, and the other strains detected in stool and sewage samples belonged to subcluster Kawasaki308 2015. The GI.3 strains detected in stool and sewage samples in the 2014-2015 and 2015-2016 seasons were included in subcluster A, whereas subcluster B included strains detected only in the sewage samples collected in the 2013-2014 season (Fig. 4B).

DISCUSSION

In the present study, we investigated the statistical relationship between the norovirus concentration in sewage and the number of reported gastroenteritis cases and also the temporal prevalence and diversity of norovirus genotypes in sewage and

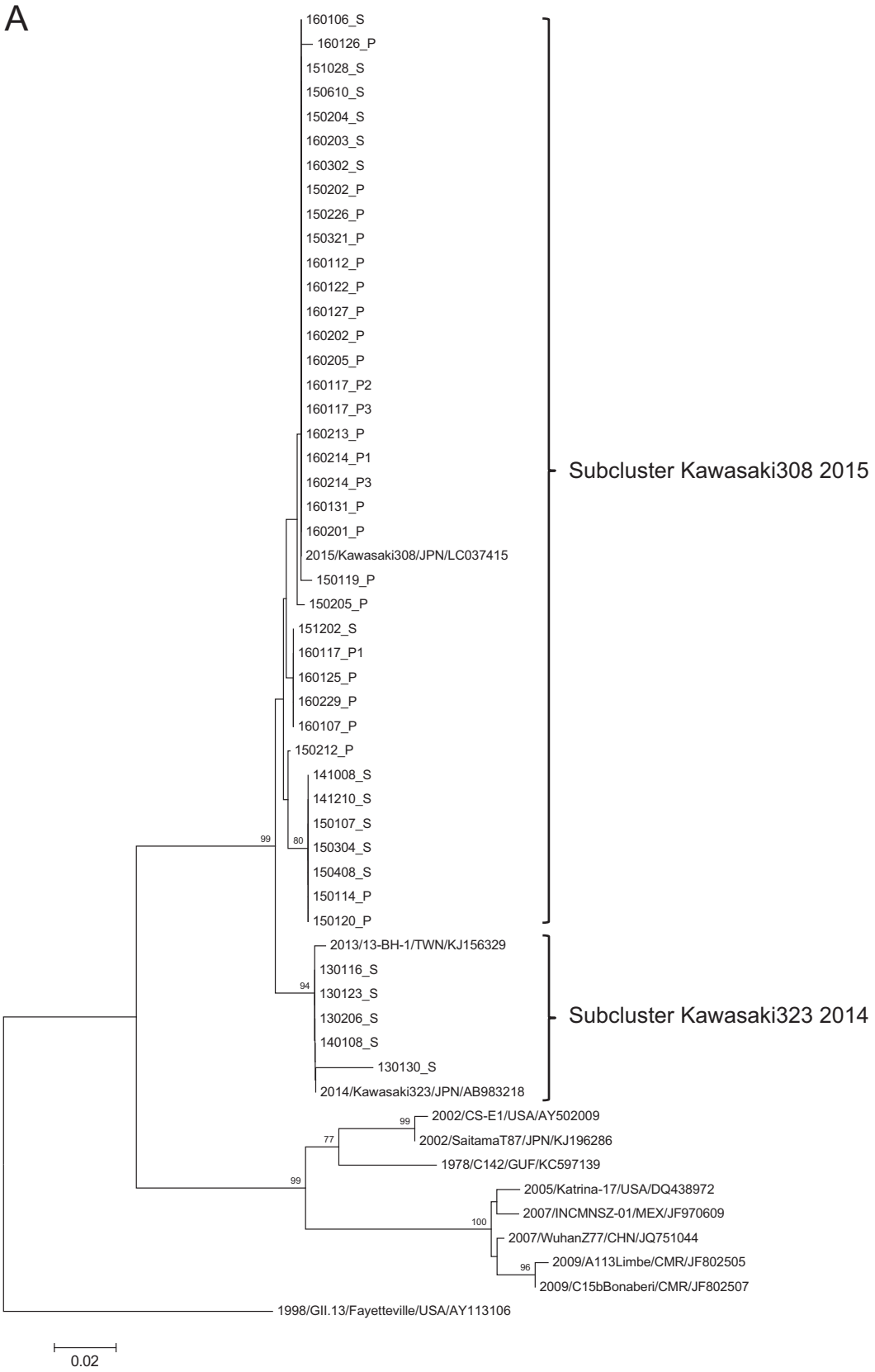

FIG 4 Phylogenetic trees of sequences obtained from sewage and stool samples (A, GII.17; B, GI.3). The name of each sequence obtained consists of the sampling date (year-month-day), followed by S (sewage) or P (patient). The name of each reference sequence consists of the year, followed by the strain name, country, and accession number. The values at the nodes represent bootstrap rates of >70%.

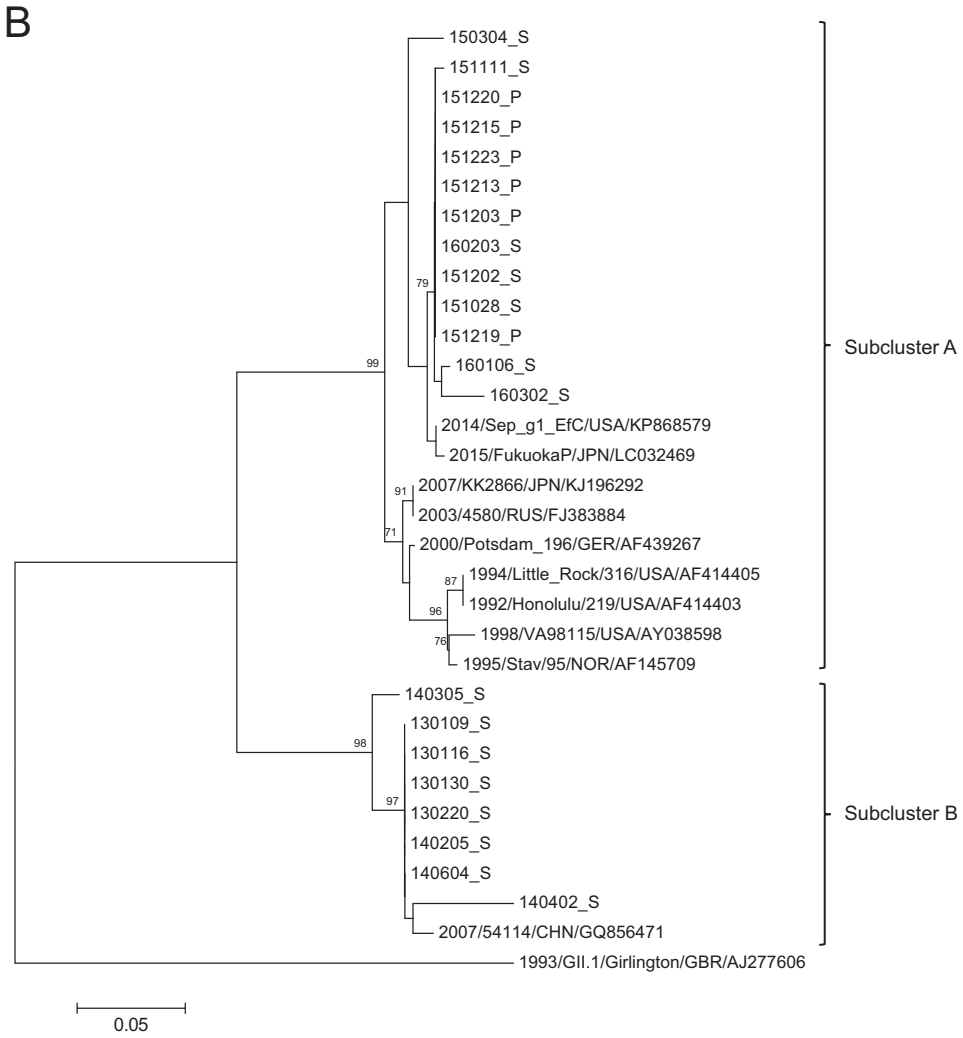

FIG 4 (Continued)

stool samples. The results of the cross-correlation analysis showed that GII concentrations in sewage significantly correlated with the number of gastroenteritis cases reported in the study area without a lag time, which suggested that monitoring of norovirus GII concentrations in sewage will be able to detect increases in norovirus infections in a catchment area. Monitoring of norovirus genotypes in sewage by pyrosequencing enabled us to understand the temporal variation of the norovirus genotypes circulating in human populations and to find that the novel GII.17 Kawasaki 2014 variant was introduced into the community in the 2012-2013 season, 1 year before it was first detected in a gastroenteritis patient in Japan. Our results suggest that monitoring of sewage for norovirus concentrations and genotype diversity can be useful for molecular epidemiological surveillance of noroviruses.

The norovirus GII concentration in sewage showed a significant correlation with the number of gastroenteritis cases reported in the study area, but the norovirus GI concentration did not. This difference is probably because the number of reported gastroenteritis cases caused by GII was significantly larger than that caused by GI. In the present study, the number of GII cases was much more than that of GI cases (92 and 14, respectively), even when the concentrations of GI and GII in sewage were comparable (e.g., January and February 2014). Previous studies also reported that GI concentrations in sewage were comparable to GII concentrations (18, 29–31), and GI cases were less frequently reported than GII cases (6, 32, 33). The highest correlation coefficient

was observed at a lag time of 0 weeks for the gastroenteritis cases reported in the study area and +5 weeks for those reported in Japan, indicating that the variation in the GII concentration in sewage was coincident with that of the gastroenteritis cases in the local population. Although a significant correlation between the GII concentration and the number of cases reported in the study area was observed at −2 to +6 weeks, the coefficients at +1 to +5 weeks were higher than those at −2 and −1 weeks. This difference is probably because the norovirus concentration in sewage reflects the number of infected individuals shedding the virus, whereas the gastroenteritis cases are counted on the basis of the date of a doctor visit. That is, the correlation with delay (lag +1 to +5 weeks) reflected norovirus shedding, which is known to continue for a few days to some months (34–37).

The GII.17 sequences obtained in our previous and present studies were clustered into two groups, subclusters Kawasaki323 2014 and Kawasaki308 2015 (10). The Kawasaki323 strain (accession number AB983218) and the Kawasaki308 strain (accession number LC037415) were identified as novel GII.P17-GII.17 variants in 2014 and 2015, respectively (12). Some sporadic cases caused by strains closely related to Kawasaki323 were reported during the 2013-2014 norovirus season in Japan (12). In our previous and present studies, the strains that belong to subcluster Kawasaki323 2014 were detected in the five sewage samples collected in the 2012-2013 and 2013-2014 seasons, although no cases caused by those strains were observed. In contrast, the strains closely related to Kawasaki308 were detected in stool samples in the 2014-2015 and 2015-2016 seasons, as well as in sewage samples. These findings suggest that the pathogenicity of these strains may be higher than that of the strains related to Kawasaki323 in the human population in the study area. The Kawasaki308 strain includes some mutations of the VP1 gene of the Kawasaki323 strain (12), and outbreak and sporadic cases were reported across the world in the 2014-2015 and 2015-2016 seasons (9–14).

We note additionally that the two GII.17 variants appeared first in sewage samples and then in stool samples. The strain closely related to the Kawasaki323 strain was first detected in a sewage sample collected on 16 January 2013 (Fig. 4A), which was 1 year earlier than its clinical identification in February 2014 in Japan (12). Similarly, the strain closely related to the Kawasaki308 strain was first detected in a sewage sample collected on 8 October 2014, and the related strain was found in a stool sample collected in February 2015 in Japan (12). Our observation clearly indicated that the novel GII.17 variants had been introduced into the community as minorities before causing an epidemic, as reported for the GII.4 Den Haag 2006b (15) and Sydney 2012 (38) variants.

Interestingly, GI.3 showed a pattern similar to that of GII.17, where GI.3 strains belonging to subcluster A were detected only in sewage samples and those belonging to subcluster B were detected in both sewage and stool samples in the 2015-2016 season. More specifically, strain 150304_S was detected in the sewage sample of March 2015 and the related strains were detected in both sewage and stool samples in the next norovirus season (Fig. 4B). Although the number of clinical samples was small, possible explanations for this result include higher pathogenicity of the strains detected in the 2015-2016 season than of those found in the 2012-2013 and 2013-2014 seasons or greater population immunity to the strains in the 2012-2013 and 2013-2014 seasons because of the cross-reactive immunity generated by other prevalent genotypes (39, 40). According to the nationwide Japanese database, 0 to 29 (mean, 8.2) cases were caused by GI.3 per year in the 2005 to 2014 seasons, but the number of cases had increased to 102 and 55 in the 2014-2015 and 2015-2016 seasons (as of November 2016), respectively (41). This fact may support our speculation. Further analysis, such as whole-genome sequencing and identification of recombination and mutations, is necessary to explain the novelty and pathogenicity of these GI.3 strains. These findings regarding GI.3, as well as GII.17, support the strong sensitivity and applicability of genotype monitoring in sewage by pyrosequencing to detect new variants that have the potential to produce an epidemic.

The sewage monitoring by pyrosequencing approach well described the diversity and temporal changes of norovirus genotypes circulating in the catchment area.

Almost all of the genotypes/variants obtained from patients were detected in sewage samples in the corresponding norovirus seasons, and those sequences were identical or very similar to each other. Such a correspondence was also observed in our previous study, where the emergence and spread of the GII.4 Sydney 2012 variant during the 2012-2013 season were observed in both sewage and stool samples (25). However, we observed discrepancies between the norovirus genotypes detected in sewage samples and those detected in stool samples. One discrepancy was that all of the samples included several genotypes for which cases were not observed during the study period. For example, 15 genotypes (18 if the genotypes detected in the previous study are included) were detected in sewage samples, whereas 4 genotypes were found in stool samples in the 2012-2013 season. Thirteen, 10, and 11 genotypes were detected in sewage samples, whereas 7, 3, and 6 genotypes were found in stool samples in the 2013-2014, 2014-2015, and 2015-2016 seasons, respectively. One explanation for this discrepancy is that most of the genotypes in the sewage samples reflected asymptomatic and/or mildly symptomatic infections. It is known that asymptomatic infections are common, and many people who are infected with norovirus do not seek medical care (1, 37, 42, 43). According to community-based studies, the monthly prevalence of norovirus in stool samples ranged from 0 to 15% and only 3% of the norovirus-positive samples were coincident with diarrhea (44), although it is not known if the prevalence of asymptomatic infections in those low-income countries is similar to that in developed countries. As a reference, in Japan, there is a report that norovirus was detected in 73% and 7% of symptomatic and asymptomatic food handlers, respectively (30). As another explanation, the number of cases in our study was observed as a part of all of the cases in the catchment area. Although the clinical importance of the genotypes or strains detected only in sewage samples may be low at that moment, their appearance in sewage indicates that viral evolution is occurring before those strains produce an epidemic. If the whole nucleotide sequence of the VP1 gene or at least the P2 subdomain region could be obtained from sewage samples, it would be informative to study the host-virus interaction of those certain genotypes or strains. Further investigation, such as cohort studies of symptomatic and asymptomatic norovirus infections, may help us understand the particular genotypes or strains detected only in sewage samples. The other discrepancy is that GII.15 and GII.21 were each detected in a single stool sample (28 July 2013 and 21 January 2016, respectively) but not in sewage samples. This discrepancy is possibly because the timing of sewage sampling (the nearest dates of the onsets are 7 August 2013 and 3 February 2016, respectively) was after the period of viral shedding by each gastroenteritis patient. Otherwise, the cases caused by those genotypes were very rare at that moment and those PCR products could not be obtained from the sewage samples.

We carried out a 3-year monitoring of norovirus in sewage and gastroenteritis cases. Previous studies (23, 27) showed that the highest concentration of norovirus in sewage was observed at a time similar to that of a peak in norovirus gastroenteritis cases. In the present study, we showed the statistical significance of the correlation between the concentration of norovirus GII in sewage and the number of gastroenteritis cases. This finding suggested that sewage monitoring for noroviruses enabled us to detect the increase in the number of infections in the catchment area, although the significance of the norovirus concentration in sewage related to the epidemic should be investigated in the future. Moreover, although there remains speculation that genotypes observed only in sewage were reflected in asymptomatic and mildly symptomatic infections, sewage monitoring by pyrosequencing was helpful in understanding the prevalence and diversity of norovirus genotypes circulating in human populations. Since human-pathogenic viruses detected in sewage are highly diverse, applying this approach to other enteric pathogens detected in sewage will enhance our understanding of their ecology.

MATERIALS AND METHODS

Study design. This study was conducted for 3 years, from April 2013 to March 2016, in the town of Matsushima, Miyagi Prefecture, Japan. As of March 2016, the town had 14,367 inhabitants and one

municipal wastewater treatment plant that received domestic wastewater from 67.4% of the population. There are four internal medicine clinics in the town, three of which participated in our virological surveillance for gastroenteritis cases. Regarding the correlation analysis of the number of gastroenteritis cases and the norovirus concentration in sewage, we used the number of gastroenteritis cases reported in the eastern area of Miyagi Prefecture, including the town of Matsushima (446,627 inhabitants as of March 2016), and that reported in Japan.

Analysis of noroviruses in stool samples. (i) Sample collection and screening. Virological surveillance for gastroenteritis cases was conducted at three outpatient clinics for internal diseases in the town. Since there is no official system of primary care by general practitioners or family physicians in Japan, patients with gastroenteritis seek a pediatrician or specialist in internal medicine. Patients with diarrhea who visited the three clinics from the 14th week of 2013 to the 13th week of 2016 were included in this study (in total 296 patients). One rectal swab sample was collected from each patient after informed consent was obtained. Since participation in this study had a minimum risk and is part of the general practice of diagnosis of gastroenteritis, we obtained verbal informed consent after explaining the study based on the consent and assent (for those <18 years old) forms. If a patient was <18 years old, informed consent was obtained from a parent or guardian. The consent was recorded in the patient's medical chart. The process by which consent was obtained was approved by the ethics committee of the Tohoku University Graduate School of Medicine. Samples were stored at 2 to 8°C and transferred to a laboratory at Tohoku University within a week. The samples were then screened for noroviruses by real-time PCR assays for norovirus GI and GII (25) within 1 to 3 days. Briefly, each rectal swab was moistened in 1 ml of phosphate-buffered saline and RNA extraction was performed with the QIAamp Viral RNA minikit (Qiagen, Hilden, Germany) with QIAcube (Qiagen). cDNA was synthesized with SuperScript III reverse transcriptase (Thermo Fisher Scientific, Waltham, MA, USA) with TaKaRa PCR Thermal Cycler Dice (TaKaRa Bio, Otsu, Japan) in accordance with the manufacturer's instructions. The amplification reaction was performed with the following primers and probes: COG1F, COG1R, RING1(1)-TP, and RING1(b)-TP for GI and COG2F, COG2R, and RING2-TP for GII (45). Noroviruses were detected by real-time PCR with the TaqMan Fast Advanced master mix (Thermo Fisher Scientific) and the Applied Biosystems 7500 real-time PCR system (Thermo Fisher Scientific) in accordance with the manufacturer's instructions (25). For reference, we also tested rotavirus, sapovirus, and astrovirus by using real-time PCR assays with previously described primers and probes (46–48).

(ii) Analysis of genotypes. Norovirus-positive rectal swab samples were assessed by Sanger sequencing. The partial RdRp and capsid N/S-encoding domain (RdRp-N/S) region of the norovirus genome in the test-positive samples was amplified by single-round PCR or nested PCR with *Ex Taq* (TaKaRa Bio, Otsu, Japan) and primers p290, COG1F, COG2F, G1SKR, and G2SKR to determine the GI and GII genotypes (45, 49, 50). The PCR products were purified with the QIAquick PCR Purification kit with QIAcube (Qiagen) or illustra ExoProStar (GE Healthcare Japan Corporation, Tokyo, Japan) and then sequenced with the BigDye Terminator v1.1 Cycle Sequencing kit and an Applied Biosystems 3130 genetic analyzer or a 3730xl DNA analyzer (Thermo Fisher Scientific) (25).

(iii) Ethics statement. This study was approved by the Research Ethics Committee of the Tohoku University Graduate School of Medicine, Sendai, Japan.

Analysis of noroviruses in sewage samples. (i) Sample collection. Primary effluents were collected at the wastewater treatment plant on Wednesdays from 3 April 2013 (the 14th week of 2013) to 30 March 2016 (the 13th week of 2016), except for the 1st week of 2014 and the last week of both 2014 and 2015; thus, 156 weekly samples were collected and analyzed. The grab samples (1 liter each) were collected around 10 a.m., transported to the laboratory on ice, and stored in a deep freezer (−80°C) until analysis.

(ii) Virus concentration, nucleic acid extraction, and RT. Noroviruses were recovered from 40 ml of the primary effluent sample and concentrated to 1 ml as virus concentrate by the PEG precipitation method (25). Prior to PEG precipitation, a known amount (approximately 10^8 genome copies) of MNV strain S7-PP3, provided by Yukinobu Tohya (Nihon University, Japan), was used to spike each sample as a whole-process control.

Viral RNA was extracted from 140 μl of the virus concentrate with the QIAamp Viral RNA minikit with QIAcube (Qiagen), and cDNA was synthesized with the iScript Advanced cDNA synthesis kit (Bio-Rad, Hercules, CA, USA) on a Veriti 96-well Thermal Cycler (Thermo Fisher Scientific) in accordance with the manufacturer's instructions (25).

(iii) Norovirus quantification by qPCR assay. Concentrations of norovirus GI and GII were determined by real-time qPCR on a CFX96 Real-Time PCR detection system (Bio-Rad). The amplification reaction was performed with SsoFast Probes Supermix (Bio-Rad) and the following primers and probes: COG1F, COG1R, RING1(a)-TP, and RING1(b)-TP for GI and COG2F, COG2R, and RING2AL-TP for GII (45, 51, 52). Each 20-μl reaction mixture contained 5 μl of cDNA, 10 μl of SsoFast Probes Supermix (Bio-Rad), and the primers and probes specified in the references. The PCR cycling conditions were 10 min at 95°C, followed by 50 cycles of 15 s at 95°C, 60 s at 56°C, and 30 s at 72°C (25).

To evaluate the overall recovery rates, the MNV used to spike each sample as a whole-process control was also quantified by qPCR (25). The overall recovery rate was calculated by dividing the copy number of the MNV detected in the virus concentrate by the copy number of the MNV used to spike the sample. MNV has been used as a process control to detect human noroviruses in drinking water (53) and sewage (54) and proposed as an appropriate control to validate the detection of norovirus in food samples (55). We used the MNV recovery rate as a quality assurance parameter only and did not use it to adjust test results (56). Samples with a recovery rate of >1.0% were adopted into norovirus quantification (57).

The number of norovirus genome copies was determined with a standard curve generated from a 10-fold serial dilution of standard DNA (10^8 to 10^1 copies/well). A synthesized oligonucleotide of the

target region (Nihon Gene Research Laboratories, Sendai, Japan) was used as the standard DNA. Each sample was measured in triplicate, and a limit for the quantification cycle value was set at 40 in accordance with the MIQE guidelines (58). The geometric mean copy number in the sample was converted to the concentration in the sewage sample only when two or more replicated quantification cycle values were <40. The norovirus genome concentrations in sewage samples were not adjusted with the MNV recovery rates.

(iv) Amplification for pyrosequencing. Norovirus analysis by pyrosequencing was performed once every 1 or 2 months (23 samples in total). Seminested PCR was performed for the selected samples with NEBNext High-Fidelity 2× PCR master mix (New England BioLabs, Ipswich, MA, USA) and 500 nM both forward and reverse primers. The primers for the first and second PCRs were COG1F/G1SKR and G1SKF/G1SKR for GI and COG2F/G2SKR and G2SKF/G2SKR for GII (45, 50). The seminested PCR products were visualized by agarose gel electrophoresis. Samples with the expected product lengths (330 and 344 bp for GI and GII, respectively) were purified and concentrated to 30 μl and then submitted for pyrosequencing (25).

(v) Pyrosequencing. To perform pyrosequencing of the norovirus amplicons with the GS Junior+ System (Roche Applied Science, Penzberg, Germany), unique adaptors were ligated to both the 5′ and 3′ ends of the amplicons by fusion PCR. Fusion PCR was carried out with 10 μl of the purified nested-PCR products. The fusion primers consisted of FLX Titanium Primer A (25-mer sequence used for sequencing), Multiplex Identifier (10-mer sequence for barcoding of each sample), G1SKF and G2SKF as forward primers, and FLX Titanium Primer B (25-mer sequence used for sequencing) and G1SKR and G2SKR as reverse primers (25).

Ninety-microliter volumes of the fusion PCR products were purified and concentrated to 30 μl with the QIAquick PCR purification kit with QIAcube (Qiagen). DNA concentrations of the purified fusion PCR products were measured with the Quant-iT PicoGreen dsDNA Assay kit (Thermo Fisher Scientific) with infinite M1000 PRO (TECAN, Männedorf, Switzerland). Four to five samples with different Multiplex Identifier sequences were mixed for the individual pyrosequencing runs with the GS Junior+ System with the Titanium emPCR kit (Lib-L) and the GS Junior Titanium Sequencing kit (Roche Applied Science) in accordance with the manufacturer's instructions (25).

(vi) Bioinformatic analysis. Bioinformatic analysis was performed with QIIME 1.8.0 software (59) as previously described (25). Briefly, quality filtering and removal of primer sequences were performed with the software package split_library.py with a minimum quality score parameter of 25. Sequences with incorrect nucleotides produced in the nested-PCR and pyrosequencing steps were corrected with the denoiser.py package. Chimeric sequences were removed with the Perseus software (60) after removing reverse primers with the split_library.py package. Sequences were then clustered into operational taxonomic units (OTUs) on the basis of a minimum nucleotide sequence similarity of 97% with the pick_otus.py package, and a representative sequence of each OTU was selected with the pick_rep_set.py package.

Genotypes and variants of the representative sequences were identified with the Norovirus Genotyping Tool Version 1.0 (61). If a given sequence could not be assigned with the Norovirus Genotyping Tool, that sequence was categorized as "not assigned." The diversity of the norovirus strains (OTUs) in each sewage sample was evaluated by using a rarefaction curve generated by the Analytic Rarefaction 2.0 software (http://strata.uga.edu/software/).

Cross-correlation analysis. The temporal relationship between the norovirus concentration in sewage and the number of infectious gastroenteritis cases reported by pediatric sentinel clinics in the study area (10 clinics) or in Japan (approximately 3,000 clinics) was evaluated by cross-correlation analysis. Since 14 October 2013, the pediatric sentinel clinics have reported gastroenteritis cases caused by rotaviruses separately. We used the data set from 16 October 2013 to 30 March 2016 (a total of 126 weeks), assuming that most of the cases were caused by noroviruses (1, 41). We performed cross-correlation analysis of the log-transformed concentration of norovirus GI or GII in sewage and the number of gastroenteritis cases by using IBM SPSS Statistics Ver. 19. We defined the lag time that the norovirus concentration changed behind as "+" and evaluated the correlation coefficients from −7 to +7 weeks with a significance level of 5%.

Phylogenetic analysis. To compare nucleotide sequences obtained from stool and sewage samples, phylogenetic analysis was carried out for all of the genotypes detected in both sewage and stool samples. Given that the sewage samples yielded several OTUs, representative sequences of OTUs containing the largest number of reads were used. In addition, sequences detected in our previous study in the 2012-2013 season (25) were also included in the phylogenic analysis. The sequences were aligned with ClustalW; bootstrapped phylogenetic trees were then constructed by the neighbor-joining method with 1,000 bootstrap replications by using the MEGA6 software (62). The genetic distances were calculated by the Kimura two-parameter method.

Accession number(s). Nucleotide sequence data from stool and sewage samples in the previous and present studies have been deposited in the DDBJ/EMBL/GenBank databases under accession numbers LC160136 to LC160238, LC169534 to LC169543, and DRA005041.

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